REPORT

EVALUATION OF THE MUTAGENIC ACTIVITY OF

[redacted]

IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY

AND THE *ESCHERICHIA COLI* REVERSE MUTATION ASSAY

(WITH INDEPENDENT REPEAT)

NOTOX Project 338737
NOTOX Substance 111834/B

CONFIDENTIALITY STATEMENT

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STATEMENT OF GLP COMPLIANCE

NOTOX B.V., 's-Hertogenbosch, The Netherlands

The study described in this report has been correctly reported and was conducted in compliance with the most recent edition of:

The OECD Principles of Good Laboratory Practice

which are essentially in conformity with:

The United States Food and Drug Administration. Title 21 Code of Federal Regulations Part 58.

The United States Environmental Protection Agency (FIFRA). Title 40 Code of Federal Regulations Part 160.

The United States Environmental Protection Agency (TSCA). Title 40 Code of Federal Regulations Part 792.

Study Director:

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Date: 11 March 2002

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Date: 11/03/2002

QUALITY ASSURANCE STATEMENT

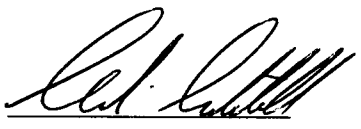
NOTOX B.V., 's-Hertogenbosch, The Netherlands

This report was audited by the NOTOX Quality Assurance Unit to ensure that the methods and results accurately reflect the raw data.

The dates of Quality Assurance inspections and audits are given below.
During the on-site inspections procedures applicable to this type of study were inspected.

DATES OF QAU INSPECTIONS/ AUDITS	REPORTING DATES
on-site inspection	
21-01-2002 to 23-01-2002 (process)	30-01-2002
protocol inspection	
21-01-2002 (study)	21-01-2002
report audit	
22-02-2002 (study)	22-02-2002

Head of Quality Assurance — C.J. Mitchell B.Sc.



Date: 13-3-02

SUMMARY

[redacted] was tested in the *Salmonella typhimurium* reverse mutation assay with four histidine-requiring strains of *Salmonella typhimurium* (TA1535, TA1537, TA100 and TA98) and in the *Escherichia coli* reverse mutation assay with a tryptophan-requiring strain of *Escherichia coli* WP_2uvrA. The test was performed in two independent experiments in the presence and absence of S9-mix (Aroclor-1254 induced rat liver S9-mix).

In the combined range finding test/first mutation assay, [redacted] was tested up to concentrations of 5000 µg/plate in the absence and presence of S9-mix. [redacted] did not precipitate on the plates at this dose level. Toxicity was observed in all tester strains.

In the second mutation assay, [redacted] was tested up to concentrations of 1000 µg/plate in the absence and presence of S9-mix in the strains TA1535, TA1537, TA98 and TA100. [redacted] was tested up to concentrations of 2000 and 1000 µg/plate in the absence and presence of S9-mix, respectively in strain WP_2uvrA. Toxicity was observed in all tester strains.

The presence of 5 and 10% (v/v) liver microsomal activation did not influence these findings.

In the second experiment in tester strain TA1537, [redacted] induced an up to 2.5-fold increase in the absence of S9-mix. However, this increase was only observed in one experiment and the highest number of revertants was not higher than 20 and within our historical control data range. Therefore, this increase is considered to be not biologically relevant and [redacted] is considered to be not mutagenic.

All other bacterial strains showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in two independently repeated experiments.

The negative and strain-specific positive control values were within our laboratory background historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

Based on the results of this study it is concluded that [redacted] is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

PREFACE

Sponsor	[redacted]
Study Monitor	Dr. C.L.J. Braun SHERA, Regulatory Affairs
Testing Facility	NOTOX B.V. Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
Study Director	C.M. Verspeek-Rip
Technical Coordinator	M.A.H. van Seggelen
Study Plan	Start : 22 January 2002 Completed : 31 January 2002

TEST SUBSTANCE

Identification	[redacted]
Chemical name	[redacted]
CAS RN	[redacted]
Description	Clear colourless liquid
Batch	1510-14
Purity	See Certificate of Analysis (Appendix 3)
Test substance storage	In refrigerator in the dark
Stability under storage conditions	Stable
Expiry date	01 January 2003
Density	Approx. 1160 $kg.m^{-3}$
Stability in vehicle	Dimethyl sulfoxide: Unknown

The sponsor is responsible for all test substance data unless determined by NOTOX.

Note: Don't heat up the test substance above 50°C

ARCHIVING

NOTOX B.V. will archive protocol, report, test article reference sample and raw data for at least 10 years. No data will be withdrawn without the sponsor's written consent.

GUIDELINES

The study procedures described in this report were based on the following guidelines:

- Organisation for Economic Co-operation and Development (OECD), OECD Guidelines for Testing of Chemicals; Guideline no. 471: "Genetic Toxicology: Bacterial Reverse Mutation Test". (adopted July 21, 1997)
- European Economic Community (EEC). Adapting to technical progress for the twenty-sixth time Annex V of the EEC Directive 67/548/EEC, Part B: Methods for the Determination of Toxicity; B.13/14: "Mutagenicity: "Reverse Mutation Assay using bacteria". EEC Publication Commission Directive (Published June 8, 2000).
- Guidelines stipulated by the Japanese Ministry of Labor and Japanese Ministry of International Trade and Industry.

OBJECTIVE

Aim of the study

The objective of this study was to evaluate the test substance for its ability to induce reverse mutations in a gene of histidine-requiring *Salmonella typhimurium* bacterial strains resulting in histidine-independent strains, and in a gene of tryptophan-requiring *Escherichia coli* bacterial strain resulting in a tryptophan-independent strain.

Background of the test system

The *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay have shown to be rapid and adequate indicators for the mutagenic activity of a wide range of chemical compounds.

The assay was conducted in the absence and presence of a metabolizing system (S9-mix).

The *Salmonella typhimurium* strains used in this study were TA98, TA100, TA1535 and TA1537. The *Escherichia coli* strain used was WP_2uvrA.

The strains TA98 and TA1537 are capable of detecting frameshift mutagens, strains TA100, TA1535 and WP_2uvrA are capable of detecting base-pair substitution mutagens (1,2,3,4 and 5).

MATERIALS AND METHODS

TEST SYSTEM

Test System	*Salmonella typhimurium* and *Escherichia coli* bacteria
Rationale	Recommended test system in international guidelines (e.g. EPA, OECD, EEC).
Source	Dr. Bruce N. Ames, University of California at Berkeley, U.S.A. (*Salmonella typhimurium* strains) TA100 received 18-02-1993, used batches: TA100.250401 and TA100.071201 TA98 received 21-02-1991, used batch: TA98.280801 TA1535 received 30-07-2001, used batch: TA1535.310701 TA1537 received 30-07-2001, used batch: TA1537.310701 Prof. Dr. B.A. Bridges, University of Sussex, Brighton, U.K. (*Escherichia coli* strain) WP_2uvrA received 23-10-1987, used batch: EC.210901

The characteristics of the different *Salmonella typhimurium* strains were as follows:

Strain	Histidine mutation	Mutation type
TA1537	*his*C3076	Frameshift
TA98	*his*D3052/R-factor*	Frameshift
TA1535	*his*G46	Base-pair substitutions
TA100	*his*G46/R-factor*	Base-pair substitutions

*: R-factor = plasmid pKM101 (increases error-prone DNA repair)

Each tester strain contained the following additional mutations:

rfa : deep rough (defective lipopolysaccharide cellcoat)
gal : mutation in the galactose metabolism
chl : mutation in nitrate reductase
bio : defective biotin synthesis
uvrB : loss of the excision repair system (deletion of the ultraviolet-repair B gene)

The *Salmonella typhimurium* strains were regularly checked to confirm their histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), UV-sensitivity and the number of spontaneous revertants.

The *Escherichia coli* WP_2uvrA strain detects base-pair substitutions. The strain lacks an excision repair system and is sensitive to agents such as UV. The strain was regularly checked to confirm the tryptophan-requirement, UV-sensitivity and the number of spontaneous revertants.

Stock cultures of the five strains were stored in liquid nitrogen (-196°C).

CELL CULTURE

Preparation of Bacterial cultures	Samples of frozen stock cultures of bacteria were transferred into enriched nutrient broth (Oxoid no. 2) and incubated in a shaking incubator (37°C, 150 spm), until the cultures reached an optical density of 1.0 ± 0.1 at 700 nm (10^9 cells/ml). Freshly grown cultures of each strain were used for a test.
Permeabilization of the *Escherichia coli* strain	WP_2uvrA bacteria were washed twice in 0.25 the original volume of ice-cold 0.12 M Tris-HCL buffer pH 8.0, then gently resuspended in 0.2 vol. 0.12 M Tris-HCL, 0.5 mM EDTA pH 8.0, and shaken for 2.5 min at 37°C. $MgCl_2$ was then added to a final concentration of 10 mM. The cells were centrifuged and resuspended in the original volume of nutrient broth.
Agar plates	Agar plates (ø 9 cm) contained 25 ml glucose agar medium. Glucose agar medium contained per liter: 18 g purified agar (Oxoid, code L28) in Vogel-Bonner Medium E, 20 g glucose. N.B. The agar plates for the test with the *Salmonella typhimurium* strains also contained 12.5 µg/plate biotin and 15 µg/plate histidine and the agar plates for the test with the *Escherichia coli* strain contained 15 µg/plate tryptophan.
Top agar	Top agar medium, containing 0.6% (w/v) agar (Oxoid no. 1) and 0.5% (w/v) NaCl, was heated to dissolve the agar. Samples of 3 ml top agar were transferred into 10 ml glass tubes with metal caps. Top agar tubes were autoclaved for 20 min at 121 ± 1 °C.
Environmental conditions	All incubations were carried out in the dark at 37 ± 1 °C. The temperature was monitored during the experiment.

TREATMENT OF THE TEST SUBSTANCE

The test substance was dissolved in dimethyl sulfoxide of spectroscopic quality (Merck). Test substance concentrations were prepared directly prior to use.

REFERENCE SUBSTANCES

Negative control

The vehicle of the test article, being dimethyl sulfoxide.

Positive controls

Without metabolic activation (-S9-mix):

Solvents for reference substances
Saline = physiological saline (NPBI, Emmer Compascuum, The Netherlands)
DMSO = dimethyl sulfoxide of spectroscopic quality (Merck).

Strain	Chemical	Concentration/plate	Solvent
TA1535	sodium azide (SA) (Sigma)	5 µg	Saline
TA1537	9-aminoacridine (9AC) (Janssen Chimica)	60 µg	Saline
TA98	daunomycine (DM) (Sigma)	4 µg	Saline
TA100	methylmethanesulfonate (MMS) (Merck)	650 µg	DMSO
WP_2uvrA	4-nitroquinoline N-oxide (4-NQO) (Sigma)	10 µg	DMSO

With metabolic activation (+S9-mix):

Strain	Chemical	Concentration/plate	Solvent
TA1537	2-aminoanthracene (2AA) (Sigma)	2.5 µg	DMSO
TA1535, TA98 and TA100	2-aminoanthracene (2AA) (Sigma)	1 µg	DMSO
WP_2uvrA[1]	2-aminoanthracene (2AA) (Sigma)	5 µg	DMSO

[1] In the presence of 10% (v/v) S9-fraction, the concentration of 2AA was 10 µg/plate.

METABOLIC ACTIVATION SYSTEM

Preparation of S9-fraction

Rat liver microsomal enzymes were routinely prepared from adult male Wistar rats, which were obtained from Charles River, Sulzfeld, Germany.

The animals were housed at NOTOX in a special room under standard laboratory conditions, as described in the Standard Operating Procedures. The rats were injected intraperitoneally with a solution (20% (w/v)) of Aroclor 1254 (500 mg/kg body weight) in corn oil. Five days later, they were killed by decapitation; (they were denied access to food for at least 12 hours preceding sacrifice). The livers of the rats were removed aseptically, and washed in cold (0°C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA. Subsequently the livers were minced in a blender and homogenized in 3 volumes of phosphate buffer with a Potter homogenizer. The homogenate was centrifuged for 15 min at 9000 g. The supernatant (S9) was transferred into sterile ampules, which were stored in liquid nitrogen (-196°C) and identified by the day of preparation.

Before use, all S9-batches were characterized with the metabolic activation requiring positive control; benzo[a]pyrene (Sigma) in tester strain TA98 at the concentration of 5 µg/plate.

Preparation of S9-mix

S9-mix was prepared immediately before use and kept on ice. S9-mix contained per 10 ml: 30 mg NADP and 15.2 mg glucose-6-phosphate in 5.5 ml or 5.0 ml Milli-Q water* (first or second experiment respectively); 2 ml 0.5 M sodium phosphate buffer pH 7.4; 1 ml 0.08 M $MgCl_2$ solution; 1 ml 0.33 M KCl solution. The above solution was filter (0.22 µm)-sterilized. To 9.5 ml of S9-mix components 0.5 ml S9-fraction was added (5% (v/v) S9-fraction) to complete the S9-mix in the first experiment and to 9.0 ml of S9-mix components 1.0 ml S9-fraction was added (10% (v/v) S9-fraction) to complete the S9-mix in the second experiment.

The S9-batch used was no. 01-10.

* Milli-Q water (Millipore Corp., Bedford, Mass., USA)

MUTATION ASSAY

Combined range finding/First experiment
Selection of an adequate range of doses was based on a range finding test in the tester strains TA1535, TA1537, TA98, TA100 and WP_2uvrA. Seven concentrations of the test substance, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate were tested in triplicate.

Second experiment
At least five different doses (increasing with approximately half-log steps) of the test substance were tested in triplicate in each strain.

The highest concentration of TRIGONOX R-938 used in the subsequent mutation assay was the level at which the test substance inhibited bacterial growth.

Experimental procedure
The test substance was tested both in the absence and presence of S9-mix in each strain, in two independent experiments.

Top agar in top agar tubes was molten and heated to 45°C. The following solutions were successively added to 3 ml molten top agar: 0.1 ml of a fresh bacterial culture (10^9 cells/ml) of one of the tester strains, 0.1 ml of a dilution of the test substance in dimethyl sulfoxide and either 0.5 ml S9-mix (in case of activation assays) or 0.5 ml 0.1 M phosphate buffer (in case of non-activation assays). The ingredients were mixed on a Vortex and the content of the top agar tube was poured onto a selective agar plate. After solidification of the top agar, the plates were turned and incubated in the dark at 37 ± 1 °C for 48 h. After this period revertant colonies (histidine independent for *Salmonella typhimurium* bacteria and tryptophan independent for *Escherichia coli*) were counted.

Colony counting
The revertant colonies (histidine independent c.q. tryptophan independent) were counted automatically with a Protos model 50000 colony counter or manually, if less than 40 colonies per plate were present.

ACCEPTABILITY OF ASSAY

A *Salmonella typhimurium* reverse mutation assay and/or *Escherichia coli* reverse mutation assay is considered acceptable if it meets the following criteria:

a) The negative control data (number of spontaneous revertants per plate) should be within the laboratory background historical range for each tester strain.

Strain		Minimum value	Maximum value	Mean ± 3 x S.D
TA1535	- S9-mix	3	23	10 ± 11
	+ S9-mix	3	23	10 ± 11
TA1537	- S9-mix	3	25	8 ± 12
	+ S9-mix	3	28	8 ± 12
TA98	- S9-mix	12	58	17 ± 16
	+ S9-mix	12	61	23 ± 21
TA100	- S9-mix	56	186	86 ± 56
	+ S9-mix	60	183	90 ± 58
WP_2uvrA	- S9-mix	4	29	12 ± 15
	+ S9-mix	4	29	12 ± 15

b) The positive control chemicals should produce responses in all tester strains which are within the laboratory historical range documented for each positive control substance.

Strain		Minimum value	Maximum value	Mean ± 3 x S.D
TA1535	- S9-mix	104	1268	253 ± 315
	+ S9-mix	50	981	262 ± 348
TA1537	- S9-mix	80	2179	339 ± 545
	+ S9-mix	73	1204	487 ± 638
TA98	- S9-mix	105	1345	477 ± 582
	+ S9-mix	97	2807	877 ± 1350
TA100	- S9-mix	201	2111	770 ± 589
	+ S9-mix	182	3435	1153 ± 1453
WP_2uvrA	- S9-mix	66	1988	633 ± 1111
	+ S9-mix	63	1532	271 ± 544

c) The selected dose range should include a clearly toxic concentration or should exhibit limited solubility as demonstrated by the preliminary toxicity range-finding test or should extend to 5 mg/plate.

DATA EVALUATION AND STATISTICAL PROCEDURES

No formal hypothesis testing was done.

A test substance is considered negative (not mutagenic) in the test if:

a) The total number of revertants in any tester strain at any concentration is not greater than two times the solvent control value, with or without metabolic activation.
b) The negative response should be reproducible in at least one independently repeated experiment.

A test substance is considered positive (mutagenic) in the test if:

a) It induces at least a 2-fold, dose related increase in the number of revertants with respect to the number induced by the solvent control in any of the tester strains, either with or without metabolic activation.
   However, any mean plate count of less than 20 is considered to be not significant.
b) The positive response should be reproducible in at least one independently repeated experiment.

The preceding criteria were not absolute and other modifying factors might enter into the final evaluation decision.

RESULTS

COMBINED RANGE FINDING/FIRST MUTATION EXPERIMENT (Table 3, Appendix 2)

[redacted] was tested in the tester strains TA1535, TA1537, TA98, TA100 and WP_2uvrA with concentrations of 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate in the absence and presence of 5% (v/v) S9-mix.

Precipitate
The test substance did not precipitate in the top agar. Precipitation of [redacted] on the plates was not observed at the start or at the end of the incubation period in all tester strains.

Toxicity
To determine the toxicity of [redacted] the reduction of the bacterial background lawn, the increase in the size of the microcolonies and the reduction of the revertant colonies were examined. The definitions are stated in Appendix 1.

The reduction of the bacterial background lawn and the reduction in the number of revertants is presented in Table 1

TABLE 1 TOXICITY OF [redacted] IN THE COMBINED RANGE FINDING/FIRST MUTATION EXPERIMENT
(Reduction of the bacterial background lawn and in the number of revertant colonies)

Strain	Without S9-mix			With S9-mix		
	Dose (µg/plate)	Bacterial background lawn	Revertant colonies	Dose (µg/plate)	Bacterial background lawn	Revertant colonies
TA1535	1000	extreme	microcolonies	1000	extreme	microcolonies
	3330-5000	absent	complete	3330-5000	absent	complete
TA1537	1000	moderate	extreme	1000	moderate	moderate
	3330-5000	absent	complete	3330-5000	absent	complete
TA98	333	slight	-[1]	1000	extreme	microcolonies
	1000	extreme	microcolonies	3330-5000	absent	complete
	3330-5000	absent	complete			
TA100	1000	extreme	microcolonies	1000	extreme	microcolonies
	3330-5000	absent	complete	3330-5000	absent	complete
WP_2uvrA	3330-5000	absent	complete	1000	extreme	microcolonies
				3330-5000	absent	complete

[1] No reduction in the number of revertants

All other concentrations, not mentioned here, showed no reduction in the bacterial background lawn and no biologically relevant reduction in the number of revertant colonies.

Mutagenicity
No biologically relevant increase in the number of revertants was observed upon treatment with [redacted] under all conditions tested.

SECOND MUTATION EXPERIMENT (Table 4, Appendix 2)

To obtain more information about the possible mutagenicity of [redacted] a second mutation experiment was performed in the absence of S9-mix and in the presence of 10% (v/v) S9-mix. The following dose range was selected for the second mutation experiment:
- TA1535, TA1537, TA98 and TA100
With and without S9-mix: 10, 33, 100, 333 and 1000 µg/plate
- WP_2uvrA
Without S9-mix: 10, 33, 100, 333, 1000 and 2000 µg/plate.
With S9-mix : 10, 33, 100, 333 and 1000 µg/plate

Precipitate
[redacted] did not precipitate in the top agar. Precipitation of [redacted] on the plates was not observed at the start or at the end of the incubation period in all tester strains.

Toxicity
The reduction of the bacterial background lawn and the reduction in the number of revertants is presented in Table 2 (See appendix 1 for definitions).

TABLE 2 TOXICITY OF [redacted] IN THE SECOND EXPERIMENT
(Reduction of the bacterial background lawn and in the number of revertant colonies)

Strain	Without S9-mix			With S9-mix		
	Dose (µg/plate)	Bacterial background lawn	Revertant colonies	Dose (µg/plate)	Bacterial background lawn	Revertant colonies
TA1535	1000	slight	-[1]	1000	extreme	microcolonies
TA1537	1000	slight	-[1]	1000	moderate	-[1]
TA98	1000	slight	slight	1000	absent	complete
TA100	1000	slight	-[1]	1000	extreme	microcolonies
WP_2uvrA	2000	absent	complete	1000	extreme	microcolonies

-[1] No reduction in the number of revertants

All other concentrations, not mentioned here, showed no reduction in the bacterial background lawn and no biologically relevant reduction in the number of revertant colonies.

Mutagenicity
In tester strain TA1537, [redacted] induced an up to 2.5-fold increase in the number of revertant colonies compared to the solvent control in the absence of S9-mix. No increase in the number of revertants was observed upon treatment with [redacted] in the other tester strains.

DISCUSSION

In the second experiment in tester strain TA1537, [redacted] induced an up to 2.5-fold increase in the absence of S9-mix. However, this increase was only observed in one experiment and the highest number of revertants was not higher than 20 and within our historical control data range. Therefore, this increase is considered to be not biologically relevant and [redacted] is considered to be not mutagenic.

All other bacterial strains showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in two independently repeated experiments.

The negative and strain-specific positive control values were within our laboratory background historical control data ranges indicating that the test conditions were adequate and that the metabolic activation system functioned properly.

CONCLUSION

Based on the results of this study it is concluded that [redacted] is not mutagenic in the *Salmonella typhimurium* reverse mutation assay and in the *Escherichia coli* reverse mutation assay.

REFERENCES

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TABLE 3 MUTAGENIC RESPONSE OF [redacted] IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY AND IN THE *ESCHERICHIA COLI* REVERSE MUTATION ASSAY

Combined range finding/first mutation experiment
Day of performance: 22 January 2002

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
	Without S9-mix				
positive control	156 ± 28	532 ± 72	454 ± 70	1099 ± 81	1054 ± 161
solvent control	7 ± 2	10 ± 4	20 ± 2	114 ± 7	19 ± 5
10	10 ± 3	6 ± 3	20 ± 6	108 ± 29	20 ± 2
33	14 ± 6	9 ± 2	18 ± 8	110 ± 15	18 ± 2
100	9 ± 2	8 ± 1	16 ± 3	131 ± 8	18 ± 2
333	8 ± 2	10 ± 3	20 ± 3 [2]	140 ± 21	15 ± 6
1000	MC [4]	3 ± 2 [3]	MC [4]	MC [4]	31 ± 5
3330	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]
5000	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]
	With S9-mix[1]				
positive control	184 ± 14	448 ± 29	504 ± 102	1239 ± 70	181 ± 23
solvent control	10 ± 1	8 ± 2	20 ± 1	84 ± 6	16 ± 3
10	12 ± 4	8 ± 4	21 ± 4	88 ± 10	17 ± 4
33	8 ± 1	12 ± 2	26 ± 10	93 ± 6	16 ± 1
100	11 ± 3	9 ± 2	28 ± 3	104 ± 9	23 ± 4
333	10 ± 4	8 ± 1	26 ± 2	127 ± 10	23 ± 6
1000	MC [4]	4 ± 4 [3]	MC [4]	MC [4]	MC [4]
3330	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]
5000	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]	0 ± 0 [5]

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 5% (v/v) S9 fraction
2 Bacterial background lawn slightly reduced
3 Bacterial background lawn moderately reduced
4 Bacterial background lawn extremely reduced
5 Bacterial background lawn absent
MC Microcolonies

TABLE 4 MUTAGENIC RESPONSE OF [redacted] IN THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY AND IN THE *ESCHERICHIA COLI* REVERSE MUTATION ASSAY

Experiment 2
Day of performance: 29 January 2002

Dose (µg/plate)	Mean number of revertant colonies/3 replicate plates (± S.D.) with different strains of *Salmonella typhimurium* and one *Escherichia coli* strain				
	TA1535	TA1537	TA98	TA100	WP_2uvrA
	Without S9-mix				
positive control	113 ± 10	512 ± 244	208 ± 11	902 ± 33	866 ± 62
solvent control	8 ± 4	6 ± 1	17 ± 4	99 ± 6	14 ± 7
10	9 ± 2	7 ± 3	15 ± 1	101 ± 8	17 ± 1
33	10 ± 1	10 ± 4	12 ± 1	111 ± 21	17 ± 3
100	13 ± 1	6 ± 2	24 ± 3	129 ± 9	13 ± 4
333	10 ± 3	12 ± 2	23 ± 6	130 ± 12	17 ± 2
1000	9 ± 3 [2]	15 ± 2 [2]	11 ± 2 [2]	132 ± 26 [2]	23 ± 3
2000					0 ± 0 [5]
	With S9-mix[1]				
positive control	98 ± 5	118 ± 28	430 ± 41	272 ± 33	163 ± 27
solvent control	7 ± 2	7 ± 3	20 ± 2	61 ± 2	15 ± 4
10	10 ± 2	7 ± 5	24 ± 6	69 ± 9	12 ± 1
33	9 ± 3	6 ± 4	22 ± 2	81 ± 9	16 ± 6
100	10 ± 4	8 ± 2	24 ± 8	70 ± 16	19 ± 4
333	10 ± 6	8 ± 1	33 ± 12	74 ± 20	28 ± 9
1000	MC [4]	5 ± 1 [3]	0 ± 0 [5]	MC [4]	MC [4]

Solvent control: 0.1 ml dimethyl sulfoxide
1 The S9-mix contained 10% (v/v) S9 fraction
2 Bacterial background lawn slightly reduced
3 Bacterial background lawn moderately reduced
4 Bacterial background lawn extremely reduced
5 Bacterial background lawn absent
MC Microcolonies

APPENDIX 1

Bacterial background lawn evaluation

The condition of the bacterial background lawn is evaluated (if indicated), both macroscopically and microscopically by using a dissecting microscope (results are normal unless indicated in tables).

Definition	Characteristics
Normal	Distinguished by a healthy microcolony lawn.
Slightly reduced	Distinguished by a slight thinning of the microcolony lawn.
Moderately reduced	Distinguished by a moderate thinning of the microcolony lawn.
Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the solvent control plate.
Absent	Distinguished by a complete lack of any microcolony background lawn.

Precipitation evaluation

Evidence of test article precipitate on the plates is recorded by addition of the following precipitation definition.

Definition	Characteristics
Slight Precipitate	Distinguished by noticeable precipitate on the plate. However, the precipitate does not influence automated counting of the plate.
Moderate Precipitate	Distinguished by a marked amount of precipitate on the plate, requiring the plate to be hand counted.
Heavy Precipitate	Distinguished by a large amount of precipitate on the plate, making the required hand count difficult.

Evaluation of the reduction in the number of revertants

The reduction in the number of revertant colonies compared to number of revertants in the solvent control is evaluated as follows:
A reduction of 21-40%: slight reduction.
A reduction of 41-60%: moderate reduction.
A reduction of 61-99%: extreme reduction.
If no revertant colonies are observed on the plates the reduction is evaluated as a complete lack of revertants.
However, any mean plate count equal to the minimal value of the historical control data range should be considered not toxic.

APPENDIX 2

Individual plate counts; (following pages)

Experiment 1
Strain TA1535

WITHOUT S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	188	139	140	156 ±	28
solvent control	5	8	7	7 ±	2
10	9	14	8	10 ±	3
33	21	13	9	14 ±	6
100	7	10	10	9 ±	2
333	6	8	9	8 ±	2
1000 [1]	MC	MC	MC	MC	
3330 [2]	0	0	0	0 ±	0
5000 [2]	0	0	0	0 ±	0

WITH S9-MIX					
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	197	184	170	184 ±	14
solvent control	9	11	10	10 ±	1
10	16	8	12	12 ±	4
33	8	7	9	8 ±	1
100	10	9	15	11 ±	3
333	11	6	14	10 ±	4
1000 [1]	MC	MC	MC	MC	
3330 [2]	0	0	0	0 ±	0
5000 [2]	0	0	0	0 ±	0

1: Bacterial background lawn extremely reduced
2: Bacterial background lawn absent
MC: Microcolonies

APPENDIX 2 – continued –

Experiment 1
Strain TA1537

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	459	533	603	532 ±	72
solvent control	14	6	11	10 ±	4
10	6	9	4	6 ±	3
33	7	10	9	9 ±	2
100	8	9	7	8 ±	1
333	13	8	10	10 ±	3
1000 [1]	1	5	2	3 ±	2
3330 [2]	0	0	0	0 ±	0
5000 [2]	0	0	0	0 ±	0

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	474	416	453	448 ±	29
solvent control	8	10	6	8 ±	2
10	9	3	11	8 ±	4
33	10	13	12	12 ±	2
100	10	10	7	9 ±	2
333	7	7	9	8 ±	1
1000 [1]	5	0	8	4 ±	4
3330 [2]	0	0	0	0 ±	0
5000 [2]	0	0	0	0 ±	0

1: Bacterial background lawn moderately reduced
2: Bacterial background lawn absent

APPENDIX 2 – continued –

Experiment 1
Strain TA98

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	510	376	476	454 ±	70
solvent control	18	19	22	20 ±	2
10	24	22	13	20 ±	6
33	27	13	15	18 ±	8
100	19	15	14	16 ±	3
333 [1]	22	16	22	20 ±	3
1000 [2]	MC	MC	MC	MC	
3330 [3]	0	0	0	0 ±	0
5000 [3]	0	0	0	0 ±	0

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	423	470	618	504 ±	102
solvent control	21	19	19	20 ±	1
10	21	24	17	21 ±	4
33	22	37	18	26 ±	10
100	25	30	29	28 ±	3
333	24	26	28	26 ±	2
1000 [2]	MC	MC	MC	MC	
3330 [3]	0	0	0	0 ±	0
5000 [3]	0	0	0	0 ±	0

1: Bacterial background lawn slightly reduced
2: Bacterial background lawn extremely reduced
3: Bacterial background lawn absent
MC: Microcolonies

APPENDIX 2 – continued –

Experiment 1
Strain TA100

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1157	1007	1134	1099 ±	81
solvent control	113	108	121	114 ±	7
10	135	113	77	108 ±	29
33	125	109	95	110 ±	15
100	136	122	135	131 ±	8
333	160	141	118	140 ±	21
1000 [1]	MC	MC	MC	MC	
3330 [2]	0	0	0	0 ±	0
5000 [2]	0	0	0	0 ±	0

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1237	1170	1309	1239 ±	70
solvent control	91	79	82	84 ±	6
10	90	77	96	88 ±	10
33	88	92	100	93 ±	6
100	101	97	115	104 ±	9
333	117	136	127	127 ±	10
1000 [1]	MC	MC	MC	MC	
3330 [2]	0	0	0	0 ±	0
5000 [2]	0	0	0	0 ±	0

1: Bacterial background lawn extremely reduced
2: Bacterial background lawn absent
MC: Microcolonies

APPENDIX 2 – continued –

Experiment 1
Strain WP_2uvrA

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	1239	943	981	1054 ±	161
solvent control	14	24	19	19 ±	5
10	21	17	21	20 ±	2
33	17	20	16	18 ±	2
100	19	19	15	18 ±	2
333	21	9	16	15 ±	6
1000	35	26	31	31 ±	5
3330 [2]	0	0	0	0 ±	0
5000 [2]	0	0	0	0 ±	0

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	174	206	162	181 ±	23
solvent control	19	17	13	16 ±	3
10	18	13	21	17 ±	4
33	15	16	17	16 ±	1
100	25	26	18	23 ±	4
333	16	25	27	23 ±	6
1000 [1]	MC	MC	MC	MC	
3330 [2]	0	0	0	0 ±	0
5000 [2]	0	0	0	0 ±	0

1: Bacterial background lawn extremely reduced
2: Bacterial background lawn absent
MC: Microcolonies

APPENDIX 2 – continued –

Experiment 2
Strain TA1535

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	123	103	114	113 ±	10
solvent control	9	4	12	8 ±	4
10	8	12	8	9 ±	2
33	11	9	11	10 ±	1
100	12	12	14	13 ±	1
333	13	9	8	10 ±	3
1000 [1]	12	6	10	9 ±	3

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	94	103	96	98 ±	5
solvent control	5	9	6	7 ±	2
10	11	10	8	10 ±	2
33	12	7	8	9 ±	3
100	15	7	8	10 ±	4
333	6	17	6	10 ±	6
1000 [2]	MC	MC	MC	MC	

1: Bacterial background lawn slightly reduced
2: Bacterial background lawn extremely reduced
MC: Microcolonies

APPENDIX 2 – continued –

Experiment 2
Strain TA1537

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	793	388	356	512 ±	244
solvent control	6	5	6	6 ±	1
10	5	5	10	7 ±	3
33	11	6	13	10 ±	4
100	4	7	6	6 ±	2
333	14	11	12	12 ±	2
1000 [1]	15	14	17	15 ±	2

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	149	113	93	118 ±	28
solvent control	4	7	9	7 ±	3
10	12	7	3	7 ±	5
33	11	4	3	6 ±	4
100	8	9	6	8 ±	2
333	7	7	9	8 ±	1
1000 [2]	4	6	4	5 ±	1

1: Bacterial background lawn slightly reduced
2: Bacterial background lawn moderately reduced

APPENDIX 2 – continued –

Experiment 2
Strain TA98

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	221	201	203	208 ±	11
solvent control	15	14	22	17 ±	4
10	14	16	14	15 ±	1
33	12	12	13	12 ±	1
100	27	22	23	24 ±	3
333	16	28	25	23 ±	6
1000 [1]	12	13	9	11 ±	2

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	435	387	468	430 ±	41
solvent control	17	21	21	20 ±	2
10	30	19	22	24 ±	6
33	21	24	22	22 ±	2
100	30	27	15	24 ±	8
333	32	21	45	33 ±	12
1000 [2]	0	0	0	0 ±	0

1: Bacterial background lawn slightly reduced
2: Bacterial background lawn absent

APPENDIX 2 – continued –

Experiment 2
Strain TA100

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	877	891	939	902 ±	33
solvent control	105	99	94	99 ±	6
10	104	92	108	101 ±	8
33	92	106	134	111 ±	21
100	127	121	139	129 ±	9
333	121	126	144	130 ±	12
1000 [1]	161	121	113	132 ±	26

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	234	290	292	272 ±	33
solvent control	63	61	59	61 ±	2
10	68	60	78	69 ±	9
33	85	87	70	81 ±	9
100	65	88	57	70 ±	16
333	57	69	96	74 ±	20
1000 [2]	MC	MC	MC	MC	

1: Bacterial background lawn slightly reduced
2: Bacterial background lawn extremely reduced
MC: Microcolonies

APPENDIX 2 – continued –

Experiment 2
Strain WP_2uvrA

	WITHOUT S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	796	893	910	866 ±	62
solvent control	19	16	6	14 ±	7
10	17	17	16	17 ±	1
33	14	19	19	17 ±	3
100	12	18	10	13 ±	4
333	15	17	18	17 ±	2
1000	22	21	27	23 ±	3
2000 [2]	0	0	0	0 ±	0

	WITH S9-MIX				
plate	1	2	3	MEAN	SD
dose (µg/plate)					
positive control	157	139	192	163 ±	27
solvent control	19	12	14	15 ±	4
10	11	12	13	12 ±	1
33	11	22	16	16 ±	6
100	22	20	14	19 ±	4
333	35	30	18	28 ±	9
1000 [1]	MC	MC	MC	MC	

1: Bacterial background lawn extremely reduced
2: Bacterial background lawn absent
MC: Microcolonies

APPENDIX 3

CERTIFICATE OF ANALYSIS

[redacted]

Certificate of Analysis [redacted]

TNA-2001007
page 1 of 2

ICS-331

Product name : [redacted]

Chemical name : [redacted]

Batch number : [redacted]

Test results:

Method	Analysis of	Unit	Result *1
Jo/72.11, Jo/95.2	Peroxidic compounds (sum) ***See page 2 for a specification***	% m/m	28.6 (± 1.5)
J20010792	Dimethyl phthalate IUPAC: Dimethyl 1.2-benzenedicarboxylate	% m/m	67.0 (± 1.0)
J20010792	IUPAC: 3-Methyl-2-butanone	% m/m	2.0 (± 0.3)
Amp/88.9	Water	% m/m	2.6 (± 0.3)
J20010792	Unidentified impurities	% m/m	0.5 (± 0.2)

*1 bracketed values are estimated 95% confidence intervals

[redacted]

APPENDIX 3 – continued –

Certificate of Analysis

specification of the peroxidic compounds

structure	% m/m

[redacted]

NOTOX Project 338737

Summarized Report of Ames Study

(Revised form for Japanese Ministry of Health & Welfare/
Japanese Ministry of International Trade & Industry)

1. General Items

Name of the new chemical substance (IUPAC) nomenclature	[redacted]		
Other name	[redacted]		
Structural formula or rational formula	[redacted] Type 3 Type 4		
[redacted]	28.6% peroxidic compounds: [redacted]		
Name and concentration of [redacted]	[redacted]		
CAS No	[redacted]		
Molecular weight	na	Vapour pressure	
Flash point	> 70 °C	Partition coefficient	Not determined
Boiling point	Na (decomposes)	Appearance at ordinary temperature	Clear and colourless liquid
Autoignation temperature	300 °C		
Stability			

Degree of Solubility	Solvent	Solubility	Stability in solvent
	Water	Immiscible	Unknown
	DMSO	>50 mg/ml	Unknown

2. Tester Strains

Strain	Obtained from	Date obtained
Salmonella typhimurium: TA98	Dr. B.N. Ames	21 February 1991
Salmonella typhimurium: TA100	Dr. B.N. Ames	18 February 1993
Salmonella typhimurium: TA1535	Dr. B.N. Ames	30 July 2001
Salmonella typhimurium: TA1537	Dr. B.N. Ames	30 July 2001
Escherichia coli: WP_2uvrA	Dr. B.A. Bridges	23 October 1987

3. S9-mix

(1) Procurement of S9

Made in-house or Purchased	Made in-house Purchase (supplier:)
Lot no. if purchased	01-10
Date of preparation	16 October 2001
Storage temperature	-196 ^{o}C

(2) Preparation of S9

Animal used		Inducing substance	
Species, Strain	Wistar	Name	Aroclor 1254
Sex	Male	Administration method	Intraperitoneally; single injection
Age (in weeks)	7 weeks	Administration period and amount (g/kg body weight)	5 days before preparing (0.5 g/kg body weight)
Weight	334-340 gram		

(3) Composition of S9 Mix

Constituents	Amount in 1 ml S9 Mix	Constituents	Amount in 1 ml S9 Mix
S9	0.05 ml (Range finding study) 0.10 ml (Main study)	NADPH	
$MgCl_2$	8 µmol	NADH	
KCl	33 µmol	Na-phosphate	100 µmol
Glucose-6-phosphate	5 µmol	Others (NADP)	3.6 µmol

4. Preparation of the Solution of the Test Substance

Solvent used	Name	Supplier	Lot no.	Grade	Purity
	Dimethyl sulfoxide (DMSO)	Merck	K27073650	Uvasol, for spectroscopy	99.8 % GC
Reason for selection of the solvent	[redacted] was insoluble in water and soluble in DMSO				
Condition of the solution of the test substance	[x] dissolved suspended others ( )				
Method of suspending when test substance is hardly soluble	not applicable				
Storage time and storage temperature during preservation until use	< 4 hour				
	Room temperature				
Correction for the purity	No				

5. Pre-culture

(1) Condition

Nutrient broth	Name	Supplier	Lot No.
	Nutrient Broth No. 2	OXOID	B: 201770
Period of preculture	5.5 hours		
Storage period/temperature from completion of incubation until use	1 to 3 hours at room temperature		
Vessel for cultivation (shape, volume)	SCOTT DURAN 100 ml erlenmeyer flask		
Container for incubation			
Volume of culture media	12 ml	Volume of the tester strain inoculated	0.1 ml

(2) Density of Tester strain Cultures at the Termination of Pre-culture

		Base-pair substitution type			Frame shift type		
		TA100	TA1535	WP_2uvrA	TA98	TA1537	
Density (x 10^9/ml)	Range finding study	1	1	1	1	1	
	Main study	1	1	1	1	1	
Method of determination	[x] 1. Conversion from the OD value 2. Dilution method 3. Others						

6. Minimum Glucose Agar Plate Medium

Made-in-house / Purchased	[Made in-house]	Purchase (supplier:)	
Date of preparation	Range finding study	Salmonella plates on Escherichia coli plates on	21 January 2002 21 January 2002
	Main study	Salmonella plates on Escherichia coli plates on	28 January 2002 21 January 2002
Lot No. (if purchased)	–		
Name of agar used Name of supplier Lot no of agar	Oxoid purified agar No. 2 OXOID (Boom B.V. Meppel, The Netherlands) 816167		

7. Test Method

(1) Test method and reason for its selection

Test method employed	Preincubation method [Direct plate method] Others
Reason	Standard Ames test

(2) Test Conditions

		Direct plate method	Preincubation method
Composition	Bacterial suspension	0.1 ml	
	Test substance solution	0.1 ml	
	Na-phosphate buffer	0.5 ml	
	S9 Mix (in case of metabolic activation method)	0.5 ml	
	Top agar solution	3 ml	
	Others ( )		
Preincubation	Temperature		
	Time		
Incubation	Temperature	37 °C	
	Time	48 hours	

8. Counting Method of the Number of Colonies

Counting Method	Manual	Colony counter
Reason for using both methods	Both methods were applied, if less than 40 colonies per plate were present, the colonies were counted manual, otherwise the colonies were counted with the colony counter	
Correction method	None	[Correction for overlapping colonies]

9. Test Result

(1) Test results are reported on the attached forms

(2) Judgement of the Results

Evaluation	Negative Positive
Reason for evaluation	In the range finding study, [redacted] was tested up to concentrations of 5000 µg/plate in the absence and presence of S9-mix. [redacted] did not precipitate on the plates at this dose level. Toxicity was observed in all tester strains. In the main study, [redacted] was tested up to concentrations of 1000 µg/plate in the absence and presence of S9-mix in the strains TA1535, TA1537, TA98 and TA100. [redacted] was tested up to concentrations of 2000 and 1000 µg/plate in the absence and presence of S9-mix, respectively in strain WP_2uvrA. Toxicity was observed in all tester strains. In the second experiment in tester strain TA1537, [redacted] induced an up to 2.5-fold increase in the absence of S9-mix. However, this increase was only observed in one experiment and the highest number of revertants was not higher than 20 and within our historical control data range. Therefore, this increase is considered to be not biologically relevant and [redacted] is considered to be not mutagenic. All other bacterial strains showed negative responses over the entire dose range, i.e. no dose-related, two-fold, increase in the number of revertants in two independently repeated experiments. The presence of 5 and 10% (v/v) liver microsomal activation did not influence these findings. Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies In conclusion, it can be stated that during the described mutagenicity test under the experimental conditions reported [redacted] did not induce point mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, [redacted] is not mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay

10. Others

Testing Institution	Name	NOTOX B.V.
	Address	Hambakenwetering 7 5231 DD 's-Hertogenbosch The Netherlands
		Tel : +31(0)73-64 06700 Fax : +31(0)73-64 06799 email : notox@notox.nl
Study director	Name and Title	C.M. Verspeek-Rip Signature: 11 March 2002
	Years of experience	17
Test date	from 22 January 2002 to 31 January 2002	
Test number.	NOTOX Project 338737 NOTOX Substance 111834/B	

APPENDIX 1

TABLE 1 TABLE OF RESULTS

Range finding study

Name of test substance: [redacted]

Date of experiment from 22 January 2002 to 24 January 2002						
With (+) or Without (-) S9-mix	Dose (µg/plate)	Number of colonies/plate (Mean of three plates)				
		Base-pair substitution type			Frame shift type	
		TA100	TA1535	WP_2uvrA	TA98	TA1537
-S9 mix	Solvent control	113, 108, 121 (114)	5, 8, 7 (7)	14, 24, 19 (19)	18, 19, 22 (20)	14, 6, 11 (10)
	10	135, 113, 77 (108)	9, 14, 8 (10)	21, 17, 21 (20)	24, 22, 13 (20)	6, 9, 4 (6)
	33	125, 109, 95 (110)	21, 13, 9 (14)	17, 20, 16 (18)	27, 13, 15 (18)	7, 10, 9 (9)
	100	136, 122, 135 (131)	7, 10, 10 (9)	19, 19, 15 (18)	19, 15, 14 (16)	8, 9, 7 (8)
	333	160, 141, 118 (140)	6, 8, 9 (8)	21, 9, 16 (15)	22, 16, 22 (20) #	13, 8, 10 (10)
	1000	MC (MC) *#	MC (MC) *#	35, 26, 31 (31)	MC (MC) *#	1, 5, 2 (3) *#
	3330	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#
	5000	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#
+S9 mix	Solvent control	91, 79, 82 (84)	9, 11, 10 (10)	19, 17, 13 (16)	21, 19, 19 (20)	8, 10, 6 (8)
	10	90, 77, 96 (88)	16, 8, 12 (12)	18, 13, 21 (17)	21, 24, 17 (21)	9, 3, 11 (8)
	33	88, 92, 100 (93)	8, 7, 9 (8)	15, 16, 17 (16)	22, 37, 18 (26)	10, 13, 12 (12)
	100	101, 97, 115 (104)	10, 9, 15 (11)	25, 26, 18 (23)	25, 30, 29 (28)	10, 10, 7 (9)
	333	117, 136, 127 (127)	11, 6, 14 (10)	16, 25, 27 (23)	24, 26, 28 (26)	7, ,7, 9 (8)
	1000	MC (MC) *#	MC (MC) *#	MC (MC) *#	MC (MC) *#	5, 0, 8 (4) *#
	3330	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#
	5000	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#	0, 0, 0 (0) *#
Positive control not requiring S9 mix	Name	MMS	SA	4-NQO	DM	9AC
	Dose (µg/plate)	650	5	10	4	60
	Number of colonies/ plate	1157, 1007, 1134 (1099)	188, 139, 140 (156)	1239, 943, 981 (1054)	510, 376, 476 (454)	459, 533, 603 (532)
Positive control requiring S9 mix	Name	2AA	2AA	2AA	2AA	2AA
	Dose (µg/plate)	1	1	5	1	2.5
	Number of colonies/ plate	1237, 1170, 1309 (1239)	197, 184, 170 (184)	174, 206, 162 (181)	423, 470, 618 (504)	474, 416, 453 (448)

MMS = methylmethanesulphonate
SA = sodium azide
4-NQO = 4-nitroquinoline N-oxide
DM = daunomycine
9AC = 9-aminoacridine
2AA = 2-aminoanthracene
MC = Microcolonies
* = Reduction in the number of revertants
\# = Reduction of the bacterial background lawn

APPENDIX 1 – continued –

TABLE 1 – continued - TABLE OF RESULTS

Main study

Name of test substance: [redacted]

Date of experiment from 29 January 2002 to 31 January 2002						
With (+) or Without (-) S9-mix	Dose (µg/plate)	Number of colonies/plate (Mean of three plates)				
		Base-pair substitution type			Frame shift type	
		TA100	TA1535	WP_2uvrA	TA98	TA1537
-S9 mix	Solvent control	105, 99, 94 (99)	9, 4, 12 (8)	19, 16, 6 (14)	15, 14, 22 (17)	6, 5, 6 (6)
	10	104, 92, 108 (101)	8, 12, 8 (9)	17, 17, 16 (17)	14, 16, 14 (15)	5, 5, 10 (7)
	33	92, 106, 134 (111)	11, 9, 11 (10)	14, 19, 19 (17)	12, 12, 13 (12)	11, 6, 13 (10)
	100	127, 121, 139 (129)	12, 12, 14 (13)	12, 18, 10 (13)	27, 22, 23 (24)	4, 7, 6 (6)
	333	121, 126, 144 (130)	13, 9, 8 (10)	15, 17, 18 (17)	16, 28, 25 (23)	14, 11, 12 (12)
	1000	161, 121, 113 (132) #	12, 6, 10 (9) #	22, 21, 27 (23)	12, 13, 9 (11) *#	15, 14, 17 (15) #
	2000			0, 0, 0 (0) *#		
+S9 mix	Solvent control	63, 61, 59 (61)	5, 9, 6 (7)	19, 12, 14 (15)	17, 21, 21 (20)	4, 7, 9 (7)
	10	68, 60, 78 (69)	11, 10, 8 (10)	11, 12, 13 (12)	30, 19, 22 (24)	12, 7, 3 (7)
	33	85, 87, 70 (81)	12, 7, 8 (9)	11, 22, 16 (16)	21, 24, 22 (22)	11, 4, 3 (6)
	100	65, 88, 57 (70)	15, 7, 8 (10)	22, 20, 14 (19)	30, 27, 15 (24)	8, 9, 6 (8)
	333	57, 69, 96 (74)	6, 17, 6 (10)	35, 30, 18 (28)	32, 21, 45 (33)	7, 7, 9 (8)
	1000	MC (MC) *#	MC (MC) *#	MC (MC) *#	0, 0, 0 (0) *#	4, 6, 4 (5) #
Positive control not requiring S9 mix	Name	MMS	SA	4-NQO	DM	9AC
	Dose (µg/plate)	650	5	10	4	60
	Number of colonies/ plate	877, 891, 939 (902)	123, 103, 114 (113)	796, 893, 910 (866)	221, 201, 203 (208)	793, 388, 356 (512)
Positive control requiring S9 mix	Name	2AA	2AA	2AA	2AA	2AA
	Dose (µg/plate)	1	1	10	1	2.5
	Number of colonies/ plate	234, 290, 292 (272)	94, 103, 96 (98)	157, 139, 192 (163)	435, 387, 468 (430)	149, 113, 93 (118)

MMS = methylmethanesulphonate
SA = sodium azide
4-NQO = 4-nitroquinoline N-oxide
DM = daunomycine
9AC = 9-aminoacridine
2AA = 2-aminoanthracene
MC = Microcolonies
* = Reduction in the number of revertants
\# = Reduction of the bacterial background lawn

APPENDIX 1 – continued –

TABLE 2 TABLE OF SPECIFIC ACTIVITY (relative to control)

	Strain	-S9 mix		+S9 mix	
		Specific activity (relative to control)	Concentration for calculation	Specific activity (relative to control)	Concentration for calculation
Range finding study	TA100	-	5000	-	5000
	TA1535	-	5000	-	5000
	WP_2uvrA	-	5000	-	5000
	TA98	-	5000	-	5000
	TA1537	-	5000	-	5000
Main study	TA100	-	1000	-	1000
	TA1535	-	1000	-	1000
	WP_2uvrA	-	1000	-	1000
	TA98	-	1000	-	1000
	TA1537	2.5-fold	1000	-	1000

- No induction in the number of revertant colonies compared to the solvent control